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Effect of Development on the Translation of Messenger RNA in *Artemia salina* Embryos*

(cysts/brine shrimp eggs/ eukaryotic initiation factors/elongation factors/brome mosaic virus RNA)

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ABSTRACT **Cell-free preparations from encapsulated *Artemia salina* embryos readily translate poly(U), but there is no endogenous protein synthesis or translation of added natural mRNA until development resumes upon incubation in saline. High-salt-washed 80S ribosomes and high-speed supernatant (cytosol) were prepared from undeveloped eggs and from eggs allowed to develop to a stage prior to hatching. Endogenous protein synthesis occurs only with ribosomes from developed embryos, whether with undeveloped or developed cytosol. This is mainly elongation of preformed polypeptide chains, for it is largely resistant to edeine, an inhibitor of chain initiation. Edeine-sensitive translation of added natural mRNA occurs with both developed and undeveloped ribosomes but requires developed cytosol. Translation of brome mosaic virus RNA is not much, if at all, further stimulated by high-salt-wash of developed ribosomes, but that of globin mRNA is markedly enhanced. Levels of the chain initiation factor EIF-1 are the same before and after development. These results are consistent with the view that resumption of development is triggered by transcription, with ensuing translation of the resulting messengers to yield, among other proteins, mRNA-recognizing initiation factors of the IF-3 type which are partly free in the cytosol and partly ribosome-bound. The data also suggest that different factors may be involved in the translation of brome mosaic virus RNA and globin mRNA by this system.**

In 1968 Hultin and Morris (3) reported that cell-free preparations from encapsulated *Artemia salina* embryos (brine shrimp eggs), which readily translate poly(U), exhibit no endogenous protein synthesis unless development resumes upon hydration of the cysts. Lack of mRNA appeared to explain the absence of protein synthesis in preparations from undeveloped cysts.

In this communication‡ we show that undeveloped *A. salina* embryos are deficient in both mRNA and one or more factors for mRNA translation, probably messenger-specific initiation factors of the IF-3 type. Two main kinds of eukaryotic initiation factors are well characterized (2): EIF-1 (M_1), the eukaryotic counterpart (4) of the bacterial initiation factor IF-2, and EIF-3 (M_3). Like IF-3, its prokaryotic equivalent (2, 5), EIF-3 is required for natural, but not for synthetic messenger translation. EIF-3's selecting for globin mRNA (6) and encephalomyocarditis virus RNA (7) have been isolated. The latter has been purified to homogeneity (8). Our results suggest that resumption of development of encysted *A. salina* embryos is triggered by transcription of embryo DNA. Translation of the resulting messengers gradually remedies the initiation factor deficiency and unlocks protein synthesis.

MATERIALS AND METHODS

Cell Fractions. Preparations of 60S and 40S ribosomal subunits, 0.5 M KCl-washed 80S ribosomes, ribosomal wash, and high-speed supernatant (s-105) fractions, from undeveloped *A. salina* cysts and cysts allowed to undergo partial development by incubation in saline, were made as described by Zasloff and Ochoa (9) with some modifications. All operations are conducted at 0°–4° and amounts given are per 100 g (dry weight) of cysts. pH values are at 25°. The cysts are stirred for 5 min with 300 ml of ice-cold NaClO, collected by filtration through a Millipore stainless steel support screen (XX20 047 08) washed 6–10 times as described (9) with 1.5 liters of ice-cold distilled water, and once with 700 ml of buffer A [20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), pH 7.6, 70 mM KCl, 9 mM $Mg(AcO)_2$, 0.1 mM ethylenediaminetetraacetate (EDTA), 1 mM dithiothreitol (DTT), 5% (v/v) glycerol]. The sedimented cysts are suspended in 900 ml of buffer A and collected by filtration, as above. The residue is transferred to a chilled mortar, mixed with 100 g of sea sand (Fisher), and thoroughly ground with the gradual addition of 100 ml of buffer A. The mixture is filtered through cheese cloth and centrifuged for 60 min at 16,000 × *g* after dilution with 60–100 ml of buffer A. The supernatant is filtered through glass wool and recentrifuged for 20 min at 30,000 × *g*. The s-30 supernatant (130–150 ml) is filtered through glass wool and centrifuged in the Spinco

Abbreviations: Designation of initiation factors of prokaryotic origin as IF-2, IF-3 and of the elongation factors of eukaryotic cytoplasmic origin as EF-1, EF-2 conforms to currently accepted nomenclature (1). So far there is no standard nomenclature for initiation factors from eukaryotic cytoplasmic sources. Factors from mammalian cells have been referred to as M_1, M_2, M_3 (2). We shall use the more general abbreviation EIF (for eukaryotic initiation factor), e.g., EIF-1, EIF-3, for these factors. In this paper the terms "embryos," "cysts," and "eggs" refer to encapsulated *Artemia salina* embryos. BMV, brome mosaic virus; $Mg(AcO)_2$, magnesium acetate; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; S-30 or -105, supernatant solutions from centrifugation at 30,000 or 105,000 × *g*. In tables, U and D are undeveloped and developed, respectively.

* *Dedicated to Otto Hoffmann-Ostenhof on his 60th birthday.*

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60 Ti rotor for 3 hr at 38,000 rpm (average 105,000 × *g*). A lipid layer is carefully removed from the surface and the top 70% of the clear, reddish-orange supernatant (80–100 ml) is collected and precipitated by addition of solid ammonium sulfate to 75% saturation. The precipitate is dissolved in a minimal volume of buffer B [20 mM Hepes, pH 7.6, 50 mM KCl, 0.1 mM EDTA, 1 mM DTT, 5% glycerol] and dialyzed against 200 volumes of buffer B for 12 hr. Any precipitate which may appear during dialysis is removed by centrifugation. This solution (A_{280}/A_{260}, about 1.2) referred to as the s-105 supernatant (or cytosol), is stored frozen at −50° in 0.3- to 0.5-ml portions; it remains fully active for about 2 months. It is a source of tRNA's, aminoacyl-tRNA synthetases, initiation, and elongation factors. The s-105 obtained from undeveloped cysts contains 0.4–0.5 g of protein (23–26 mg/ml), the corresponding solution from developed cysts contains 0.6–0.8 g of protein (30–40 mg/ml).

The ribosomal pellets are used for preparation of either subunits as described (9) or 0.5 M KCl-washed 80S ribosomes and ribosomal wash. The pellet is carefully rinsed with buffer A, stirred in buffer C [20 mM Hepes, pH 7.6, 0.5 M KCl, 5 mM $Mg(AcO)_2$, 0.1 mM EDTA, 2 mM DTT, 5% glycerol] for 12 hr with a magnetic stirrer and centrifuged in the Spinco no. 30 rotor for 40 min at 26,000 rpm. The top two-thirds (the clear portion) of the supernatant is collected and centrifuged in the Spinco 60 Ti rotor for 5 hr at 38,000 rpm. The clear supernatant is precipitated with ammonium sulfate at 75% saturation, dissolved in a minimal volume of buffer C, and dialyzed for 12 hr against 200 volumes of the same buffer. The resulting solution ($A_{280/260}$, 0.95–1.0), which contains a total of 40–100 mg of protein (6–10 mg/ml), will be referred to as the ribosomal wash. It is stored frozen in small aliquots at −50°. The stability of this preparation is similar to that of the s-105 supernatant. After removing by decantation the remaining supernatant, the ribosomal pellet is rinsed with buffer D [20 mM Hepes, pH 7.6, 100 mM KCl, 5 mM $Mg(AcO)_2$, 0.1 mM EDTA, 1 mM DTT, 5% glycerol], suspended in buffer D to a volume of about 6 ml, layered over 26 ml of 25% sucrose in the same buffer, using Spinco polycarbonate tubes, and centrifuged in the Spinco 60 Ti rotor for 12 hr at 38,000 rpm. The clear, colorless ribosomal pellet is suspended (see ref. 9) in buffer D containing 50% (v/v) glycerol, to a concentration of 400 A_{260} units/ml, and stored at −20°. The bulk of the high-salt-washed ribosomes consists of 80S monomers. They are referred to as ribosomes. They retain activity for several months.

Development of Cysts. The cysts (100 g dry weight) carried through the last washing with distilled water are suspended in 5 liters of 4% NaCl and incubated with vigorous aeration for 16 hr at 27°. Little or no hatching occurs during this time. The mixture is chilled in ice, the embryos are allowed to settle, and the turbid supernatant, containing considerable debris, is carefully decanted. The embryos are washed 5–6 times with 2–2.5 liters of ice-cold distilled water until the washes are virtually salt-free. The wash fluid is removed each time by decantation after allowing the cysts to settle. The residue from the last water wash is filtered through a Millipore stainless steel support screen, suspended in 700 ml of buffer A, and processed for extraction as described above.

Incubations and Assays. Unless otherwise noted, samples contained, in a final volume of 0.1 ml, Hepes buffer, pH 7.6, 20 mM; KCl, 100 mM; $Mg(AcO)_2$, 3 mM; DTT, 1 mM; ATP, 1.3 mM; GTP, 0.33 mM; phosphocreatine, 10 mM; creatine kinase, 6.6 μg (0.9 unit); tRNA (*Escherichia coli* W), 0.5 A_{260} unit; L-[^{14}C]leucine (specific radioactivity, 640 cpm/pmole), 0.02 mM; the remaining 19 L-amino acids, each 0.05 mM; ribosomes, 2 A_{260} units (about 0.2 mg); s-105 supernatant, about 0.7 mg of protein; and, when present, ribosomal wash, 60–70 μg of protein, and mRNA [rabbit globin or brome mosaic virus (BMV) RNA], about 7 μg. This amount was found to be optimal in the case of BMV RNA. Although tRNA was routinely added, the amount present in the s-105 supernatants is apparently adequate, since similar incorporation values are obtained without or with addition of *E. coli*, rat liver, or yeast tRNA. An inhibitor of translation is present in the cytosol of *A. salina* embryos. It is soluble in 75% saturated ammonium sulfate and is largely removed by ammonium sulfate precipitation of the active components during preparation of the s-105 supernatant fractions (M. Zasloff and S. Ochoa, unpublished observations). However, these fractions still contain some inhibitory material and the same is true of the high-salt ribosomal wash fractions. The source of ribosomes, s-105 supernatant, and ribosomal wash used, whether from undeveloped (U) or developed (D) *A. salina* cysts, is indicated in the tables.

TABLE 1.
Endogenous mRNA translation by A. salina ribosomes

Ribosomes	s-105 supernatant	[^{14}C]Leucine incorporation (pmole)
None	U	1.1
None	D	1.2
U	None	0.2
U	U	1.2
U	D	1.2
D	None	0.8
D	U	14.3
D	D	10.6

Nineteen nonlabeled amino acids, each 0.02 mM; s-105 supernatant with 1 mg of protein. Incubation, 60 min at 28°.

The reaction components are added at 0° in the order listed above, except for [^{14}C]leucine which is added last. Incubation is for 45–60 min at 30°. The incorporation of labeled amino acid proceeds at a constant rate for about 30 min and drops rather sharply thereafter. For determining the incorporation of radioactivity into acid-insoluble material, reactions are terminated by addition of 2 ml of 10% trichloroacetic acid followed by heating at 90° for 20 min and cooling. The samples are filtered on Whatman GF/C filters, and the precipitates washed with 50 ml of 5% trichloroacetic acid, dried, and counted in Omnifluor in a Packard-Tri-Carb liquid scintillation spectrometer at 86% efficiency. Protein was determined by the Lowry procedure (10) with bovine-serum albumin as standard.

Preparations. Brine shrimp eggs were purchased from Longlife Aquarium Products, Harrison, N. J. Rabbit globin mRNA was obtained from the Searle Laboratories, High Wycombe, Bucks, England. BMV was kindly provided by Dr. Paul Kaesberg, University of Wisconsin, Madison, Wisc. Edeine A_1 was a gift from Drs. W. Szer and Z. Kurylo-Borowska.

TABLE 2. *BMV RNA translation by A. salina ribosomes*

Sample	Ribosomes	s-105 supernatant	Ribosomal wash	[14C]Leucine incorporation (pmole) Endogenous (a)	With BMV RNA (b)	Net (b − a)
1	U	None	None	0.3*		
2	D	None	None	0.5*	0.4*	0.0
3	None	U	U	1.3		
4	None	D	D	1.4		
5	U	U	None	1.3*	1.3	0.0
6	U	U	U	1.2*	1.2	0.0
7	D	U	None	8.2		
8	D	U	U	6.7		
9	D	D	None	8.4*	14.5*	6.1
10	D	D	D	7.8*	15.0*	7.2
11	U	D	None	1.8*	9.9	8.1
12	U	D	D	1.4*	7.7	6.3

* Average of duplicate runs.

BMV RNA was isolated from the virus by the phenol extraction procedure (11). *A. salina* EIF-1 (highly purified) was provided by Dr. C. Nombela of this department. Other preparations were as in previous work (9).

RESULTS

Endogenous mRNA Translation. As seen in Table 1 there is little incorporation of [14C]leucine into acid-insoluble material with undeveloped ribosomes, regardless of whether the s-105 supernatant (cytosol) is derived from undeveloped or developed cysts. On the other hand, developed ribosomes show good endogenous incorporation with undeveloped or developed cytosol.

Translation of Added mRNA. A system of undeveloped ribosomes and cytosol fails to translate added natural mRNA. Table 2 illustrates this point for BMV RNA (samples *5* and *6*). On the other hand, a system with developed cytosol readily translates the added messenger whether with undeveloped (Table 2, samples *11* and *12*) or developed (samples *9* and *10*) ribosomes. These results cannot be explained by the presence of an inhibitor in undeveloped and its absence from developed cytosol because the former does not prevent the stimulatory effect of the latter. Note that addition of developed ribosomal wash does not enhance or enhances only slightly the translation of either endogenous mRNA or BMV RNA (samples *9–12*). Similar results are obtained with globin mRNA (Table 3) except that translation of this messenger is enhanced by developed ribosomal wash. As with BMV RNA, there is no translation of the added messenger with

TABLE 3. *Globin mRNA translation by A. salina ribosomes*

Sample	Ribosomes	s-105 supernatant	Ribosomal wash	[14C]Leucine incorporation (pmole) Endogenous (a)	With globin RNA (b)	Net (b − a)
1	D	D	None	7.4	8.4	1.0
2	D	D	D	5.6	11.2	5.6
3	U	D	None	1.9	6.7	4.8
4	U	D	D	1.5	10.0	8.5
5	U	None	None	0.3		
6	U	U	D	1.3	2.7	1.4
7	U	U	None	1.3	1.4	0.1
8	U	D	None	1.9	7.2	5.3
9	U	D	D	1.5	10.5	9.0

undeveloped cytosol and ribosomes (sample *7*) but there is translation with developed cytosol, whether with undeveloped (samples *3* and *4*, *8* and *9*) or developed (samples *1* and *2*) ribosomes. In all cases developed ribosomal wash enhances globin mRNA translation. In Table 4, with undeveloped ribosomes and developed cytosol, developed ribosomal wash is seen once more not to affect BMV RNA translation (10.1 pmoles without, 9.9 pmoles with wash) but to increase the translation of globin mRNA (7.6 pmoles without, 10.9 p-moles with).

Effect of Edeine on Translation of Endogenous and Added mRNA. The effect of edeine, a potent inhibitor of polypeptide chain initiation (9, 12), was investigated on endogenous and added messenger translation in the *A. salina* system. As seen in Table 5, endogenous mRNA translation with undeveloped cytosol is not significantly inhibited by edeine at the concentration used, but translation of added messenger, whether BMV RNA (experiment *1*) or globin mRNA (experiment *2*) is strongly inhibited. Endogenous translation with developed cytosol is significantly (30–35%) inhibited by edeine. Thus, the observed endogenous incorporation of [14C]leucine into material insoluble in hot trichloroacetic acid is largely due to chain elongation on polyribosomes formed during development of the embryos. These polysomes appear not to be disrupted by high-salt washing of the ribosomal pellets.

DISCUSSION

Eukaryotic cells can translate a variety of heterologous messengers (2, 13–17). In the *A. salina* system we have thus far used BMV RNA and globin mRNA. Both messengers are translated with fidelity by wheat germ or embryo systems (18, 19). BMV RNA is also translated by L cells (20) and by

TABLE 4. *Translation of BMV RNA and globin mRNA by A. salina ribosomes*

Ribosomes	s-105 supernatant	Ribosomal wash	[14C]Leucine incorporation (pmole) Endogenous (a)	With BMV RNA (b)	Net (b − a)	With globin mRNA (c)	Net (c − a)
U	D	None	2.1	12.2	10.1	9.7	7.6
U	D	D	1.6	11.5	9.9	12.5	10.9

TABLE 5. *Effect of edeine on mRNA translation of A. salina ribosomes*

Exp. No.	mRNA	Ribosomes	s-105 supernatant	Edeine*	[14C]Leucine incorporation (pmole) Endogenous (a)	With added mRNA (b)	Net (b − a)
1	None	D	U	−	11.3		
	None	D	U	+	10.3		
	BMV	D	D	−	15.4	21.1	5.7
	BMV	D	D	+	10.2	10.1	0
	BMV	U	D	−	2.1	14.5	12.4
	BMV	U	D	+	0.7	1.0	0.3
2	Globin	D	D	−	14.3	18.8	4.5
	Globin	D	D	+	9.9	9.7	0
	Globin	U	D	−	1.7	11.8	10.1
	Globin	U	D	+	0.8	1.2	0.4

* 0.9 μM.

ascites s-30 extracts (B. V. Treadwell and W. G. Robinson, personal communication).

Our results support the following conclusions: (*1*) Undeveloped *A. salina* cysts are deficient in factors required for translation of mRNA. This is shown by the fact that added mRNA is not translated by undeveloped ribosomes with undeveloped cytosol. Factors formed during cyst development are found in developed cytosol and, to some extent, in developed ribosomal wash. (*2*) Undeveloped cysts are also deficient in mRNA. This follows from the fact that there is little endogenous incorporation by *undeveloped* ribosomes with *developed* cytosol, a system which readily translates added mRNA. (*3*) The occurrence of chain elongation with *developed* ribosomes, whether with *undeveloped* or *developed* cytosol, indicates that mRNA (and polysomes) is formed upon cyst development. (*4*) Since there is chain elongation on endogenous mRNA (*developed* ribosomes) in the presence of undeveloped cytosol, the required enzymes, i.e., elongation and release factors and aminoacyl-tRNA synthetases, are not limiting in undeveloped cysts. This suggests that these cysts are deficient, besides mRNA, in one or more initiation factors. (*5*) As shown in Table 6, cytosols from developed and undeveloped cysts have equal amounts of EIF-1. This suggests that the initiation factors that are deficient in undeveloped cysts and are produced during cyst development are of the messenger-selecting EIF-3 type. (*6*) The fact that factors produced during development, promoting BMV RNA translation, are mainly restricted to the cytosol, whereas factors promoting globin mRNA translation are present in both developed cytosol and ribosomal wash suggests, but does not prove, that translation of these messengers may involve different EIF-3's. Multiplicity of EIF-3's is suggested by the isolation from ascites cell cytosol of a factor, IF_{EMC}, which promotes encephalomyocarditis virus RNA, but not globin, mRNA translation (7, 8). Factors (M_3) promoting globin mRNA translation are also known (6, 21).

The above facts are consistent with the view that resumption of cyst development upon hydration is triggered by transcription of embryo DNA with ensuing translation of the resulting messengers. This yields, among other proteins, mRNA-selecting factors of the IF-3 type. The initiation factor deficiency is thus gradually corrected and protein synthesis is unlocked.

TABLE 6. *EIF-1 levels in supernatant from undeveloped and developed A. salina cysts*

Additions			f[14C]Met-tRNA bound (pmole)		
AUG (A_{260} units)	Supernatant (μg of protein)	EIF-1 (μg)	Total	Net due to EIF-1	EIF-1 specific activity*
—	—	1.2	0.14		
0.05	—	—	0.55		
0.05	—	1.2	5.57	5.02	4200
0.05	(U) pH 5 (105)	—	1.02	0.47	4.5
0.05	(D) pH 5 (105)	—	1.00	0.45	4.3
0.05	(U) AS (189)	—	2.53	1.98	10.5
0.05	(D) AS (180)	—	2.55	2.00	11.1

EIF-1 in supernatant fractions was assayed, through its requirement for the AUG-dependent binding of f[14C]Met-tRNA to *A. salina* 40S ribosomal subunits at 0°, as previously described (4). The supernatant fractions from undeveloped (*U*) or developed (*D*) cysts, corresponded to steps 2 (pH 5.3 supernatant, abbreviated pH 5) and 3 (ammonium sulfate fractionation, abbreviated *AS*) of the EIF-1 purification procedure of Zasloff and Ochoa, and were prepared as described (4).

* pmoles of f[14C]Met-tRNA bound per mg of protein.

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